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Original Article

TOTAL PHENOL, FLAVONOID AND ANTIOXIDANT PROPERTIES OF *AURICULARIA AURICULA-JUDAE*

PACKIALAKSHMI B.[a]*, SUDHA G.[b], CHARUMATHY M.[a]

[a]Department of Biochemistry, Kongunadu Arts and Science College, Coimbatore 641029, Tamil Nadu, India, [b]Department of Biochemistry, School of Biosciences, Periyar University, Salem 636011, Tamil Nadu, India
Email: biochem_bagya@yahoo.co.in

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ABSTRACT

Objective: From ancient times, *Auricularia auricula* has been used as food and also as herbal medicine in China. The hot water extract of *Auricularia auricula* fruiting bodies were evaluated for free radical scavenging potency, total phenol and flavonoid content.

Methods: The antioxidant property was investigated using different *in vitro* assays such as, ABTS (2,2'-azino-bis[3-ethylbenzothiazoline-6-sulphonicacid]), DMPD (Dimethyl-4-phenylenediamine) radical scavenging assay, reducing power, phenanthroline assay, lipid peroxidation and erythrocyte hemolysis inhibition assays and also subjected to analyse total phenol, flavonoid levels and total antioxidant capacity by phospho-molybdenum assay.

Results: The levels of total phenol and flavonoid were found to be 8.94 mg CE/g, 3.49 mg RE/g respectively. The EC_{50} values are<10 mg/ml in all the assays which indicate its strong scavenging activities. The antioxidant activity was found to be concentration-dependent. A significant difference ($P<0.05$) was observed between the concentrations tested.

Conclusion: These results suggest that *Auricularia auricula* mushrooms are good scavengers of ABTS, DMPD radicals, reducer of ferric ions, and inhibitor of lipid peroxidation and erythrocyte hemolysis. Thus, it could potentially be used as a source of bioactive compounds.

Keywords: Antioxidant activity, Black fungus, Erythrocyte hemolysis, Free radicals, Lipid peroxidation, Phytochemicals.

INTRODUCTION

Free radicals or reactive oxygen species (ROS) are defined as any molecules or atoms with one or more unpaired electrons, usually being unstable and highly reactive. ROS, such as superoxide anion radical ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2), hydroxyl radical ($OH^{\cdot}$) and other free radicals are byproducts of biological metabolism and are known to induce lipid peroxidation, damage of lipids, proteins and nucleic acids in cells [1] and also related to aging and diseases such as rheumatoid arthritis, emphysema, atherosclerosis and cancer [2]. Antioxidants can scavenge free radicals and provide protection against diseases. Although almost all organisms possess antioxidant defense and repair systems that have evolved to protect them against oxidative damage, these systems are not sufficient to prevent the damage entirely. Thus, it is essential to explore new natural antioxidants to protect human from free radicals and abate the progression of many chronic diseases.

In recent years, mushrooms are used in various health care food and medicine, and it have drawn the attention of nutritional and biochemical researchers because of their various biological activities [3]. Mushrooms such as *Phellinus ribis, Agaricus brasiliensis, Agaricus bisporus, Ganoderma lucidum* and *Phellinus linteus, Pleurotus ostreatus, Calocybe gambosa, Clitocybe odora, Coprinus comatus, Armillaria mellea* and *Ganoderma atrum* have been investigated for their antioxidant properties and are used for the treatment of various diseases [4, 5].

The *Auricularia* mushroom species are the fourth most important cultivated mushrooms used by people throughout the world [6]. *Auricularia auricula-judae* belongs to Auriculariaceae family, commonly known as "Tree-ear" or "wood ear" with a gelatinous texture. It has been reported that it's fruiting bodies are rich in carbohydrates, proteins, alkaloids, vitamins, minerals (Ca, Fe and P) and has a low-fat content [7]. It's fruiting bodies exhibit various pharmacological activities including immunomodulating, antinociceptive, anticancer, hypolipidemic and hypoglycemic [8]. There are not many investigations about the free radical scavenging mechanism of *Auricularia auricula-judae* using different *in vitro* assays. The aim of the present study was to evaluate the antioxidant activities in hot water extract from *Auricularia auricula* fruiting bodies and to study the phytochemical components.

MATERIALS AND METHODS

Chemicals and reagents

2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonicacid) (ABTS), Dimethyl-4-phenylenediamine (DMPD) and 2,2'-Azobis(2-amidinopropane) dihydrochloride (AAPH) were obtained from Sigma-Aldrich (Bangalore, India). BHT (Butylated hydroxytoluene), gallic acid, catechin, ascorbic acid, linoleic acid and thiobarbituric acid (TBA) were obtained from Himedia (Mumbai, India). All other chemicals are of analytical grade.

Mushroom samples

The dried fruiting bodies of *Auricularia auricula-judae* (888) were obtained from Hangzhou Haudan Agri-food mushroom farm, Hangzhou City, Zhejiang Province, China. The dried fruiting bodies were powdered (20 mesh) and stored in air-tight plastic bags for further analysis.

Preparation of the extracts

Mushroom powder (10 g) was extracted by stirring with 100 ml of boiling water at 100 °C for 3 h. After centrifugation at 5000 g for 20 min, the residues were re-extracted twice with the boiling water. The supernatants were pooled together, and the combined extracts were evaporated under reduced pressure at 45 °C for 30 min using a rotary vacuum evaporator. The extract obtained was dissolved in hot water at 100 mg/ml. From the stock solution, successive dilutions were made and used for various *in vitro* assays to analyze the antioxidant activity of the samples. Analyses were carried out in triplicates.

Estimation of total phenol

The total phenol in the mushroom extract was measured according to the method of [9] with some modifications. 1.0 ml of the sample was mixed with 1.0 ml of Folin-Ciocalteu's phenol reagent. After 3 min, 1.0 ml of saturated sodium carbonate (35%) was added to the mixture and it was made up to 10 ml by adding deionized water. The mixture was kept for 90 min at room temperature in the dark. The

absorbance was measured at 725 nm against the blank. Pyrocatechol was used as the reference standard. The total phenol content is expressed as milligrams of catechol equivalents (CE) per gram of extract.

Estimation of total flavonoid

Total flavonoid content was determined as described by [10]. 0.25 ml of mushroom extract was diluted with 1.25 ml of distilled water. 75 µl of a 5% sodium nitrite were added and after 6 min 150 µl of a 10% aluminum chloride were added and mixed. After 5 min, 0.5 ml of 1 M sodium hydroxide was added. The absorbance was measured immediately against the prepared blank at 510 nm. Rutin was used as the reference standard. The total flavonoid content is expressed as milligrams of rutin equivalents (RE) per gram of extract.

Total antioxidant capacity by Phospho molybdenum assay

The antioxidant activity of the sample was evaluated by the phospho- molybdenum method according to the procedure of [11]. An aliquot of 0.1 ml of sample solution was mixed with 1.0 ml of the reagent solution (0.6 M sulphuric acid, 28 mM sodium phosphate, and 4 mM ammonium molybdate). The tubes were capped with silver foil and incubated at 95 °C for 90 min. The tubes were cooled to room temperature and the absorbance of the sample was measured at 695 nm against a blank. Ascorbic acid was used as a standard and total antioxidant capacity was expressed as milligrams of ascorbic acid equivalents (AAE) per gram of extract.

ABTS radical cation scavenging activity

The ABTS radical cation scavenging activity was performed with slight modifications described by [12]. The $ABTS^{\cdot+}$cation radicals were produced by the reaction between 7 mM ABTS in water and 2.45 mM potassium persulfate, stored in the dark at room temperature for 12 h. Prior to use, the solution was diluted with ethanol to get an absorbance of 0.700±0.025 at 734 nm. Free radical scavenging activity was assessed by mixing 10 µl of the test sample with 1.0 ml of ABTS working standard in a microcuvette. The decrease in absorbance was measured exactly after 6 min. The percentage inhibition was calculated according to the formula: $[(A_0-A_1)/A_0] \times 100$, where A_0 was the absorbance of the control, and A_1 was the absorbance of the sample. Ascorbic acid was used as a positive control.

Determination of reducing power

The reducing power of hot water extract was measured according to the method of Oyaizu [13]. The reaction mixture contained 2.5 ml of various concentrations of the extracts, 2.5 ml phosphate buffer (0.2 M, pH 6.6) and 2.5 ml of 1% potassium ferricyanide were mixed and incubated at 50 °C for 20 min and centrifuged for 10 min at 5000 g after addition of 2.5 ml of 10% TCA. To 2.5 ml aliquot of the supernatant, 2.5 ml of deionized water and 0.5 ml of 0.1% ferric chloride were added and mixed well. After 10 min of incubation, the absorbance was measured at 700 nm against a blank. Ascorbic acid was used as a positive control.

DMPD radical scavenging activity

The principle of $DMPD^{\cdot+}$assay is that at acidic pH and in the presence of a suitable oxidant solution, DMPD can form a stable and coloured radical cation ($DMPD^{\cdot+}$). The assay was performed according to the method of [14, 15]. $DMPD^{\cdot+}$was obtained by adding 0.2 ml ferric chloride (0.05 M) to DMPD in acetate buffer. 0.5 ml of various concentrations of the extract and 1 ml of $DMPD^{\cdot+}$solution were vortexed, and incubated in dark at room temperature for 10 min. The absorbance was measured at 505 nm. The buffer solution was used as a blank sample. The scavenging activity was calculated using the following equation: $(A_0-A_1/A_0) \times 100$, where A_0 was the absorbance of the control and A_1 was the absorbance of the sample. Ascorbic acid was used as a positive control.

Phenanthroline assay

The phenanthroline assay was used to determine the reducing capacity of mushroom extract, according to the method of [16]. Various concentrations of the sample (0.10 ml), ferric chloride (0.50 ml, 0.2%) and 0.5% of 1, 10-phenanthroline solution (0.25 ml) were mixed, and made up the volume to 5 ml with methanol. The reaction mixture was then incubated at 30 °C in the dark for 20 min and the absorbance orange-red solutions was measured at 510 nm against a reagent blank. BHT was used as a positive control.

Lipid peroxidation inhibition assay

A modified thiobarbituric acid reactive species (TBARS) assay [17, 18] was used to measure the lipid peroxide formed, using rat liver homogenate. Malondialdehyde has been identified as the product of lipid peroxidation that reacts with thiobarbituric acid to give a red colour absorbing at 535 nm. To 1.0 ml of extract, add 1.0 ml of 1% liver homogenate, then 0.05 ml of 0.5 mM $FeCl_2$ and 0.5 mM H_2O_2 were added to initiate lipid peroxidation. After incubation at 37 °C for 60 min, 1.5 ml of 20% TCA and 1.5 ml of 0.8% TBA solution (0.8%, w/v) were added to quench the reaction. The resulting mixture was heated at 100 °C for 15 min and then centrifuged at 4000 rpm for 10 min. The absorbance of the upper layer was measured at 532 nm. The inhibition effect on lipid peroxidation was calculated as follows: Inhibition effect (%) = $[1–(A_1-A_2)/A_0] \times 100$, where A_0 was the absorbance of the control (water instead of sample), A_1 was the absorbance of the sample, and A_2 was the absorbance of the sample only (water instead of liver homogenate). Ascorbic acid was used as a positive control.

Inhibition of erythrocyte hemolysis

The inhibition of erythrocyte hemolysis mediated by peroxyl free radicals by the mushroom extract was determined according to the procedure described by [19]. Blood was obtained from the rat. Erythrocytes were separated from the plasma and the buffy coat were washed three times with 10 ml of 10 mM PBS, pH 7.4 and centrifuged at 1500 g for 5 min. During the last washing, the erythrocytes were obtained by centrifugation at 1500 g for 10 min. A suspension of erythrocytes in PBS (20%, 0.1 ml) was added to 0.2 ml of 200 mM AAPH solution in PBS, and 0.1 ml of various concentrations of the sample. The reaction mixture was shaken gently (30 rpm) while being incubated at 37 °C for 3 h. The reaction mixture was diluted with PBS (8 ml) and centrifuged at 3000 g for 10 min; the absorbance of its supernatant was then read at 540 nm. The percentage hemolysis inhibition was calculated by the equation: [(AAAPH-AS)/AAAPH] × 100, where AS was the absorbance of the sample and AAAPH was the absorbance of the control sample. Ascorbic acid was used as a positive control.

Statistical analysis

All assays were carried out in triplicates and results are expressed as mean±SD. The data were analysed using SPSS software. Analysis of variance (ANOVA) and Duncan's multiple range test (DMRT) were used to analyze the differences among scavenging activity and EC_{50} of various extracts for different antioxidant assays with least significance difference (LSD) $P<0.05$ as a level of significance.

RESULTS AND DISCUSSION

Total phenol, flavonoid content and total antioxidant capacity assay

Phenolic compounds such as flavonoids, phenolic acids and tannins are considered to be major contributors to the antioxidant capacity of plants. These antioxidants also possess various biological activities, such as anti-carcinogenic, anti-inflammatory, and anti-atherosclerotic activities [20]. Therefore, the total phenolic content is important for the antioxidant activity of mushroom extracts [21]. Flavonoids are the most common and widely distributed group of plant phenolics and are very effective antioxidant compounds [22]. Table 1 represents the total phenol and flavonoid content of *Auricularia auricula* extract. The total phenolic and flavonoid content was found to be 8.94 mg CE/g, 3.49 mg RE/g respectively.

The total antioxidant capacity (TAC) assay is based on the reduction of Mo (V) to Mo (IV) by the antioxidants in the sample and subsequent formation of a green phosphate/Mo(V) complex at acidic pH with an absorbance maximum at 695 nm [23]. The total antioxidant capacity *A. auricula* hot water extract was found to be 7.60 mg AAE/g (table 1).

Table 1: Total phenol, flavonoid content and total antioxidant capacity of *Auricularia auricula* hot water extract[#]

Sample	Total Phenols (mg CE/g)[A]	Total Flavonoids (mg RE/g)[B]	Total antioxidant capacity (mg AAE/g)[C]
A. auricula	8.94±0.52	3.49±0.26	7.6±0.37

[#]Values are expressed as mean±SD (n = 3). [A]CE = catechol equivalents; [B]RE = rutin equivalents; [C]AAE = ascorbic acid equivalents.

ABTS radical scavenging assay

ABTS$^{\bullet+}$radicals are highly reactive radicals and unlike the reactions with DPPH radical, which involve H-atom transfer, the reactions with ABTS$^{\bullet+}$radicals involve an electron transfer process [24]. It is one of the spectrophotometric methods that have been applied to the measurement of the total antioxidant activity of beverages, pure substances and aqueous mixtures [25]. It is a decolorization technique, in which the radical is generated directly in a stable form when it reacts with antioxidants in the sample. Bleaching of a preformed solution of the blue-green radical cation ABTS$^{\bullet+}$has been used to analyse the antioxidant potency of complex mixtures and individual substances**.** *Auricularia auricula* hot water extract showed ABTS radical scavenging effect in a concentration-dependent manner (fig. 1). The ABTS radical scavenging ability was between 50.57-75.46% at 4-20 mg/ml. A significant difference (P<0.05) was found between the different concentrations tested. EC_{50} values of ABTS radical scavenging activity was found to be in the order: ascorbic acid (0.20 mg/ml)>*A. auricula* (4.40 mg/ml). These results indicated its stronger scavenging activity on ABTS radical.

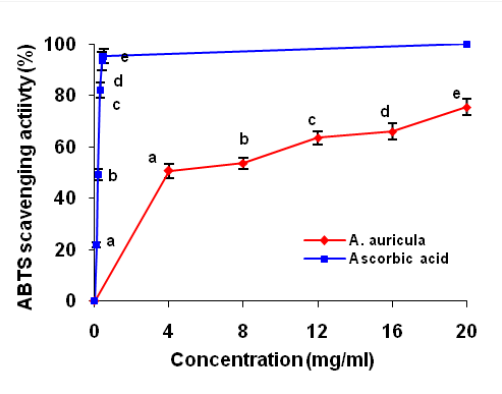

Fig. 1: ABTS radical scavenging activity of hot water extract of *A. auricula* and standard ascorbic acid at different concentrations. Results are expressed as mean±SD (n = 3). Different letters (a-e) indicate a significant difference between the concentrations of the same extract (P<0.05, ANOVA, DMRT)

Reducing power

The reduction capacity of a compound may serve as a significant indicator of its potential antioxidant activity. Antioxidant compounds are able to donate electrons to reactive free radicals and reduce into more unreactive and stable species [26]. The reducing power of *A. auricula* extract increased as the concentration increased from 1 to 5 mg/ml. At 1-5 mg/ml, the reducing powers of *A. auricula* were 0.267-1.106 and at 0.2-1.0 mg/ml, the reducing powers of ascorbic acid were 0.295-1.213 (fig. 2). The EC_{50} value was found to be 2.20 mg/ml for *A. auricula* and 0.38 mg/ml for ascorbic acid. Statistically significant difference was found between the concentrations tested (P<0.05). The reducing properties of the sample are generally associated with the presence of reductones which could react with the radicals to stabilize and terminate free radical reactions. Our data on reducing power showed that the hot water extract from *A. auricula* can act as electron donors and can act on free radicals to convert them into more stable products.

DMPD radical scavenging assay

The principle of this assay is that, DMPD can form a stable and coloured radical cation (DMPD$^{\bullet+}$) at acidic pH with the presence of a suitable oxidant solution. The maximum absorbance can be measured at 505 nm. Antioxidant compounds, which are able to transfer hydrogen atom to DMPD$^{\bullet+}$and leads to decolouration of the solution. Decolourisation is the end point of the reaction, which is rapid and stable and is considered as a measure of the antioxidant capacity. Therefore, this assay shows the ability of radical hydrogen donors to scavenge the single electron from DMPD$^{\bullet+}$ [27]. At 2-10 mg/ml, the DMPD radical scavenging ability of *A. auricula* was between 44.05 to 78.15% (fig. 3) whereas DMPD radical scavenging ability of ascorbic acid was between 20.67 to 82.09% at 0.04 to 0.2 mg/ml and the EC_{50} values were ranked in the order: ascorbic acid (0.011 mg/ml)>*A. auricula* (2.05 mg/ml). A significant difference (P<0.05) was found between the different concentrations tested. These results suggest that the extracts are capable of scavenging DMPD radicals.

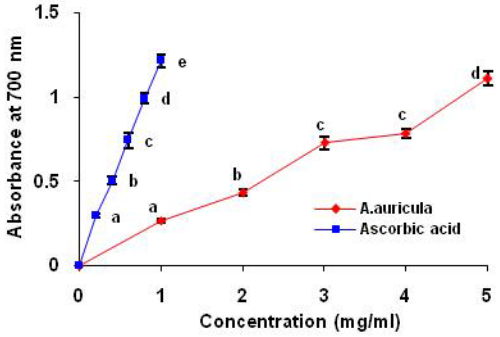

Fig. 2: Reducing power of hot water extract of *A. auricula* and standard ascorbic acid at different concentrations. Results are expressed as mean±SD (n = 3). Different letters (a-e) indicate a significant difference between the concentrations of the same extract (P<0.05, ANOVA, DMRT)

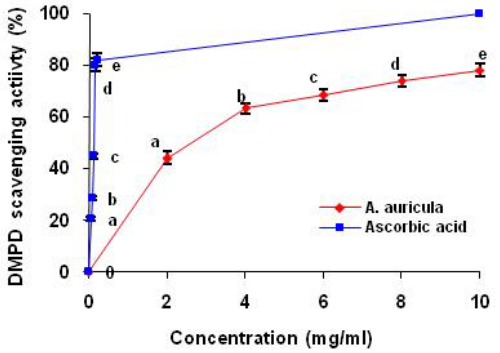

Fig. 3: DMPD radical scavenging activity of hot water extract of *A. auricula* and standard ascorbic acid at different concentrations. Results are expressed as mean±SD (n = 3). Different letters (a-e) indicate a significant difference between the concentrations of the same extract (P<0.05, ANOVA, DMRT)

Phenanthroline assay

The phenanthroline method is based on the ability of antioxidants to reduce Fe (III) to Fe (II) [28]. This change in absorbance can be measured at 510 nm. The scavengers in the extract interfered with

the ferrous-o-phenanthroline complex formation, thereby suggesting that the extract has metal chelating capacity. The reducing abilities increased with the increase of sample concentration (fig. 4). At the concentration of 2-10 mg/ml, the reducing ability of *A. auricula* extract were between 0.432-0.807 whereas BHT showed better reducing the ability of 0.554 to 1.435 at 0.2-1.0 mg/ml. Iron reducing ability was in the following order: BHT (EC_{50} = 0.15 mg/ml)>*A. auricula* (EC_{50} = 3.3 mg/ml). A statistically significant difference (P<0.05) in iron-reducing ability was observed with the concentrations tested.

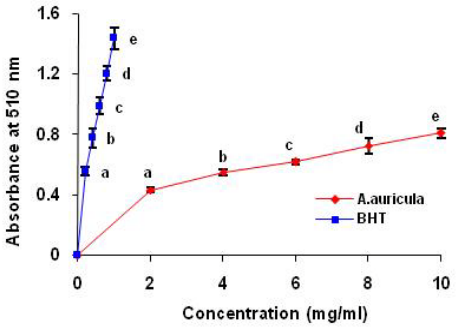

Fig. 4: Phenanthroline assay of hot water extract of *A. auricula* and standard BHT at different concentrations. Results are expressed as mean±SD (n = 3). Different letters (a-e) indicate a significant difference between the concentrations of the same extract (P<0.05, ANOVA, DMRT)

Lipid peroxidation inhibition assay

The cell membrane's fluidity and permeability are maintained by its glycerides, fatty acids, and phospholipids composition. This could be affected by free radicals by inducing lipid peroxidation [29]. LPO inactivates cellular components and plays a key role in oxidative stress in biological systems. Transition metal ions, such as iron and copper, are known to stimulate LPO through various mechanisms. Lipid peroxidation inhibition could be assessed by the amount of MDA produced. Thus, the decrease in the MDA level with the increase in the concentration of the extract indicates the role of the extract as an effective antioxidant (fig. 5) and there was a significant difference between the concentrations tested (P<0.05). LPO inhibition strongly increased from 18.95 to 54.90% when the concentration of the extract increased from 1 to 5 mg/ml and exhibited EC_{50} value of 3.89 mg/ml whereas the EC_{50} value of ascorbic acid was found to be 0.022 mg/ml.

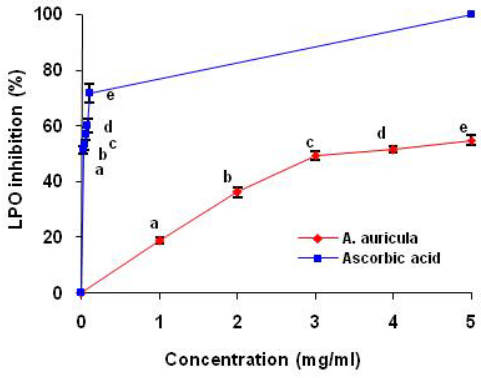

Fig. 5: Lipid peroxidation inhibition of hot water extract of *A. auricula* and standard ascorbic acid at different concentrations. Results are expressed as mean±SD (n = 3). Different letters (a-e) indicate a significant difference between the concentrations of the same extract (P<0.05, ANOVA, DMRT)

Erythrocyte hemolysis inhibition

To elucidate the biological relevance of the antioxidant activities of crude polysaccharides from *Auricularia* mushrooms, the human erythrocyte was used herein as a cell-based model system. The AAPH induced oxidative damage on human erythrocytes has been extensively studied as a model for per oxidative injury in biological membranes [30-32] leading to hemolysis. The extract inhibited hemolysis in a concentration-dependent manner (fig. 6). The inhibition of erythrocyte hemolysis of *A. auricula* was between 6.67-59.37% at 2-10 mg/ml. The hierarchy of inhibition was in the order: ascorbic acid (EC_{50} = 0.17 mg/ml)>*A. auricula* (EC_{50} = 9.01 mg/ml). A significant difference (P<0.05) was found in the EC_{50} value between different concentrations of *Auricularia auricula* extract tested. This study suggests that *A. auricula* extract effectively inhibited erythrocyte hemolysis in *in vitro* condition.

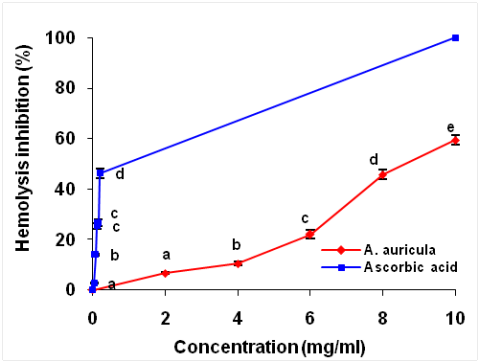

Fig. 6: Erythrocyte hemolysis inhibition of hot water extract of *A. auricula* and standard ascorbic acid at different concentrations. Results are expressed as mean±SD (n = 3). Different letters (a-e) indicate a significant difference between the concentrations of the same extract (P<0.05, ANOVA, DMRT)

CONCLUSION

On the basis of the results, it is concluded that the hot water extract from *Auricularia auricula* were found to possess radical scavenging and antioxidant activities. Effectiveness in antioxidant activity is inversely correlated to EC_{50} values. Substantial amounts of phenols and flavonoids were present. These results will be of great significance to further utilize this mushroom resource. However, further studies are necessary for the pharmacological activity of *Auricularia auricula* extracts *in vivo*.

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CONFLICT OF INTERESTS

The authors have no conflict of interest

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